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Rapid Flow Cytometry Method for Quantitation of LFA-1-Adhesive T Cells

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Adhesion molecules are important for leukocyte endothelial attachment and migration to sites of inflammation. The LFA-1 (CD11a and CD18) integrin molecule is constitutively expressed on the T-cell surface. Following T-cell activation, a rapid conformational change of LFA-1 to an "adhesive" state occurs, allowing LFA-1 binding to intracellular cell adhesion molecule type 1 (ICAM-1)-expressing targets, such as antigen-presenting cells. For this study, a rapid flow cytometry method for the quantitation of LFA-1-adhesive T cells following activation was developed. Purified ICAM-1 was bound to 4.5-μm-diameter beads. Following peripheral blood mononuclear cell activation culture (phorbol myristate acetate and ionomycin), the cells were incubated with the ICAM-1 beads, which allowed attachment to occur. The T cell-bead complexes were then resolved from unbound T cells by flow cytometry. Multicolor analysis allowed a complete phenotypic analysis of the adhesive T-cell subsets. Experimental controls indicated that the T cell-bead attachment was LFA-1 and ICAM-1 specific. Very little binding between unactivated T cells and ICAM beads or between activated T cells and plain beads was observed. The kinetics of the response was extremely rapid, with nearly maximal numbers of adhesive T cells observed following 5 min of activation. Scanning electron microscopy analysis was used to characterize legitimate bead-cell binding. By using multicolor cytometry, the responding adhesive T-cell population was usually identified as a distinct subset of T cells with the following phenotype: $CD3^+$ $CD4^+$ or $CD8^+$ $CD19^-$ $CD16^-$ $CD45RO^+$ $CD62L^+$ $CD27^+$ $CD57^-$. A rapid and simple method for the scoring of LFA-1-adhesive T cells was developed and may have significant utility for immune function studies.

Adhesion molecules are membrane-bound proteins present on the surfaces of cells that regulate both cell-cell interactions and the interaction between cells and the extracellular matrix. All stages of leukocyte migration (rolling, adhesion, and extravasation) are influenced by specific leukocyte adhesion molecules (7). In particular, T-lymphocyte migration is important to give the small numbers of T lymphocytes specific for any particular antigen the best chance to encounter that antigen. Lymphatic drainage and cell migration ensure that lymphocytes, antigen-presenting cells, and foreign antigen converge in the lymph nodes, whereas blood-borne pathogens are usually eliminated by the spleen. The extravasation of lymphocytes is controlled ("lymphocyte homing") so that appropriate subsets of lymphocytes leave the circulation and enter the correct tissues. Naïve or resting T cells tend to migrate through high endothelial venules into secondary lymphoid tissues, while activated T cells tend to migrate to sites of inflammation. High endothelial venules express on their luminal surfaces vascular homing receptors that bind to T-cell counterreceptors.

Integrin adhesion molecules, expressed on leukocytes and other cell types, are heterodomeric cell surface molecules consisting of a variety of alpha and beta chains. They bind to cell adhesion molecules (CAMs) belonging to the immunoglobulin superfamily. The T cell LFA-1 integrin is composed of both an alpha chain (CD11a) and a beta chain (CD18). The ligand for LFA-1 is the intracellular cell adhesion molecule type 1 (ICAM-1 or CD54).

The control that adhesion molecules exert over leukocyte migration is influenced by both the number and the type of adhesion molecules present and the activation state of the leukocyte. Some adhesion molecules (including LFA-1) are constitutively expressed at high levels, but cellular activation induces a conformation change in the protein from a "nonadhesive" to an "adhesive" state (1, 3, 5, 9, 10). A number of mitogenic stimuli may regulate integrin adhesivness, including pharmacological agents such as phorbol ester and Ca^{2+} ionophore (2, 3, 5, 6), or ligand engagement of the T-cell receptor (3, 5, 6). Activation-dependent regulation of integrin-mediated adhesion is important for T-cell migration and recognition of foreign antigen (8). Alternatively, many cells retain large stores of adhesion molecules in vesicles, which can be directed to the cell surface within minutes following cellular activation. New molecules can also be synthesized and transported to the cell surface, a process which usually takes several hours.

This study confirmed that the LFA-1 molecule is constitutively expressed on the surfaces of T cells at a relatively high level and that surface LFA-1 levels do not change following T-cell activation with a variety of mitogens. It may then be inferred that LFA-1 adhesiveness is changed following T-cell activation in some relevant T-cell subsets. An attempt was made to develop a rapid and accurate flow cytometry-based method for the detection of LFA-1-adhesive T cells that would be compatible with multiparameter analysis for other simultaneous assessments, such as phenotype analysis.

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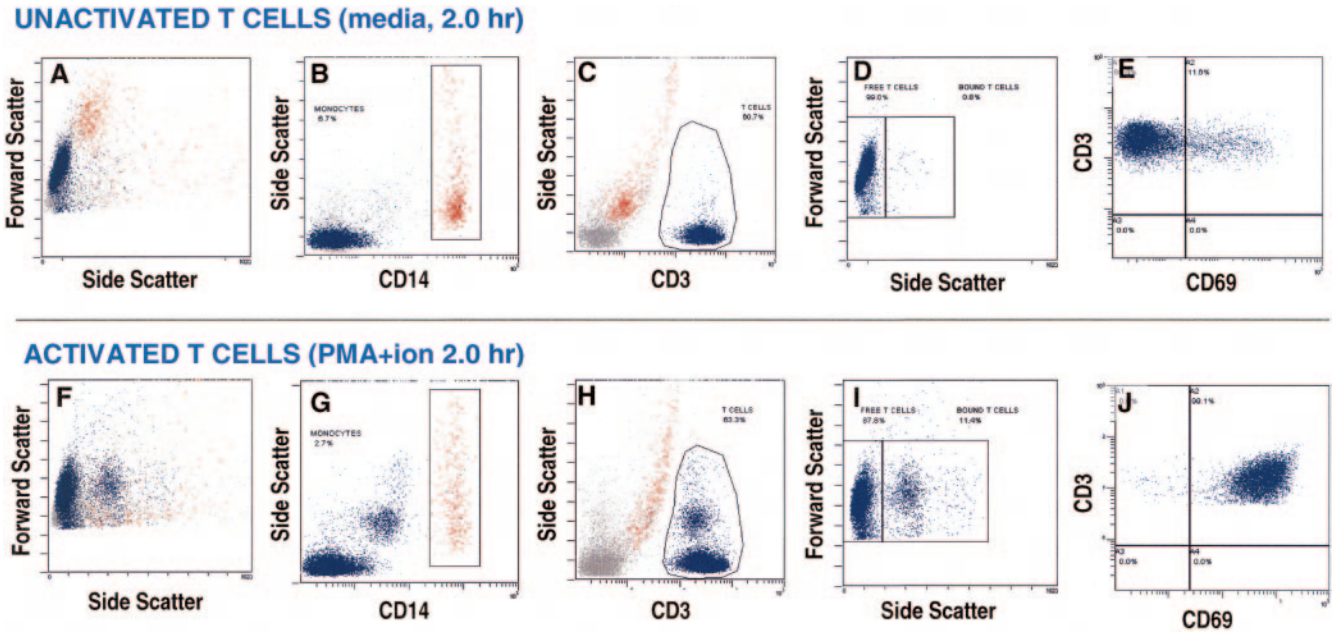

FIG. 1. Primary scatter plot. Quantitation of activated T-cell binding to ICAM-1 beads and potential interference by monocytes and T-cell activation (CD69 expression) were also simultaneously assessed via multicolor flow cytometry. PBMCs were cultured for 2 h in medium alone (unactivated; upper row) or in medium containing PMA-ionomycin (activated; lower row). Monocytes are gated via detection of CD14 expression (B and G) and tracked as red (all plots). Note the presence of monocytes in the unactivated cultures scatter plot (A) but their relative absence in the stimulated culture scatter plot (F). The population in panel F that appears to be monocytes is in fact T cell-bead complexes. T cells (bound or unbound to beads) were gated by CD3 expression (C and H) and then plotted alone versus side scatter for quantitation of T cell-bead complexes (D and I). T cell-bead complexes were resolved via the increase in scatter properties, corresponding to the conversion of LFA-1 to an adhesive state and bead binding. Note the relative absence of LFA-1-adhesive T cells in the unstimulated culture (D) and the defined population in the activated culture (I). T-cell activation was simultaneously tracked by measurement of CD69 expression for both the unstimulated culture (E) and activated culture (J). The activated T cells in the unstimulated culture (E) represent normal constitutive levels of CD69 expression.

MATERIALS AND METHODS

Blood donors. For this assay development activity, whole-blood samples were obtained from adult donors and placed into acid citrate dextrose anticoagulant Vacutainers. The subjects had been screened by the NASA-JSC Test Subject Facility for exposure to most major infectious diseases. The approval of the NASA-JSC Institutional Review Board (Committee for Protection of Human Subjects) was obtained for this study, and written informed consent was obtained from all subjects.

Creation of ICAM-1 beads. To create ICAM-1 beads, 40 μl of anti-mouse immunoglobulin G (IgG) magnetic beads (Dynal Biotech, Lake Success, NY) was washed three times, according to the manufacturer's instructions. A total of 50 μl of mouse anti-human Fc antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA) was added to the washed beads, and the mixture was incubated for 15 min to allow binding. The resulting anti-human Fc beads were then washed three times and incubated with 25 μg of ICAM-1 Fc chimera protein (R&D Systems Inc., Minneapolis, MN) for 1 h. Following incubation, 20 μl of mouse serum (Organon Teknika, Durham, NC) was added to block the unbound anti-Fc binding sites on the beads, and the incubation was continued for an additional 30 min. The resulting ICAM-1 beads were then washed three times and resuspended in 200 μl of phosphate-buffered saline. The beads were stored at 4°C until use in an experiment. Control beads were created by processing the beads exactly as described above, but the addition of the ICAM-Fc chimera protein was eliminated.

Cell culture and staining. Purified peripheral blood mononuclear cells (PBMCs) were used for all activation cultures. PBMCs were created by the Ficoll gradient separation of approximately 7.0 ml of acid citrate dextrose-anticoagulated whole blood. For culture activation, the PBMCs were resuspended at 1×10^6 cells per 1.0 ml of medium containing 10 ng/ml phorbol myristate acetate (PMA) and 5.0 μg/ml ionomycin for the appropriate length of time (between 30 s and 3.0 h, as indicated). Following incubation, the activated cells were analyzed immediately by the direct addition of the ICAM-1 beads without washing. The bead-cell mixture was incubated for a minimum of 20 min at 4°C to prevent further T-cell activation. The samples were then analyzed directly by flow cytometry.

Flow cytometry analysis of T cell-bead complexes. A flow cytometry protocol that allowed gating of T cells and T cell-bead complexes, tracking of monocytes, and assessment of activation and associated surface markers was devised (Fig. 1). Briefly, flow cytometry samples were stained according to the antibody manufacturer's instructions (Beckman Coulter, Miami, FL). The antibody matrix consisted of CD3 (fluorescein isothiocyanate [FITC])-CD69 (phycoerythrin [PE])-CD14 (phycoerythrin-cyanin 5 [PC5]). The surface markers were interchanged as required for further phenotypic analysis of responding T cells.

ESEM analysis. For environmental scanning electron microscope (ESEM) analysis, the cells were fixed in paraformaldehyde, followed by postfixation with 0.1 mM cacodylic acid–0.1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA). The samples were then sequentially dehydrated with increasing concentrations of ethanol (at 25%, 50%, 60%, 70%, 80%, and 90% and twice at 100%), sputter coated and analyzed with an FEI/Philips XL30 environmental scanning electron microscope in the environmental mode.

Confocal microscopy. Immunofluorescent analysis of cell-bead interactions was carried out on a Bio-Rad Radiance 2100 confocal microscope. Briefly, cells labeled with FITC–LFA-1 were incubated with ICAM-1-expressing magnetic beads, followed by fixation in 1% paraformaldehyde. The samples were then bound on polyethylenimine-coated slides, followed by incubation with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR) to enhance the signal with a more stable fluorophore. Vectashield (Vector Laboratories, Burlingame, CA) was added to preserve the fluorescence, and the samples were analyzed by confocal microscopy with a krypton-argon laser.

RESULTS

Assessment of constitutive T-cell LFA-1 expression. All peripheral T cells were found to constitutively express LFA-1 protein. Mitogenic stimulation of T cells during culture with either CD3-CD28 or PMA-ionomycin did not appreciably raise or lower the levels of LFA-1 protein on the T-cell surface.

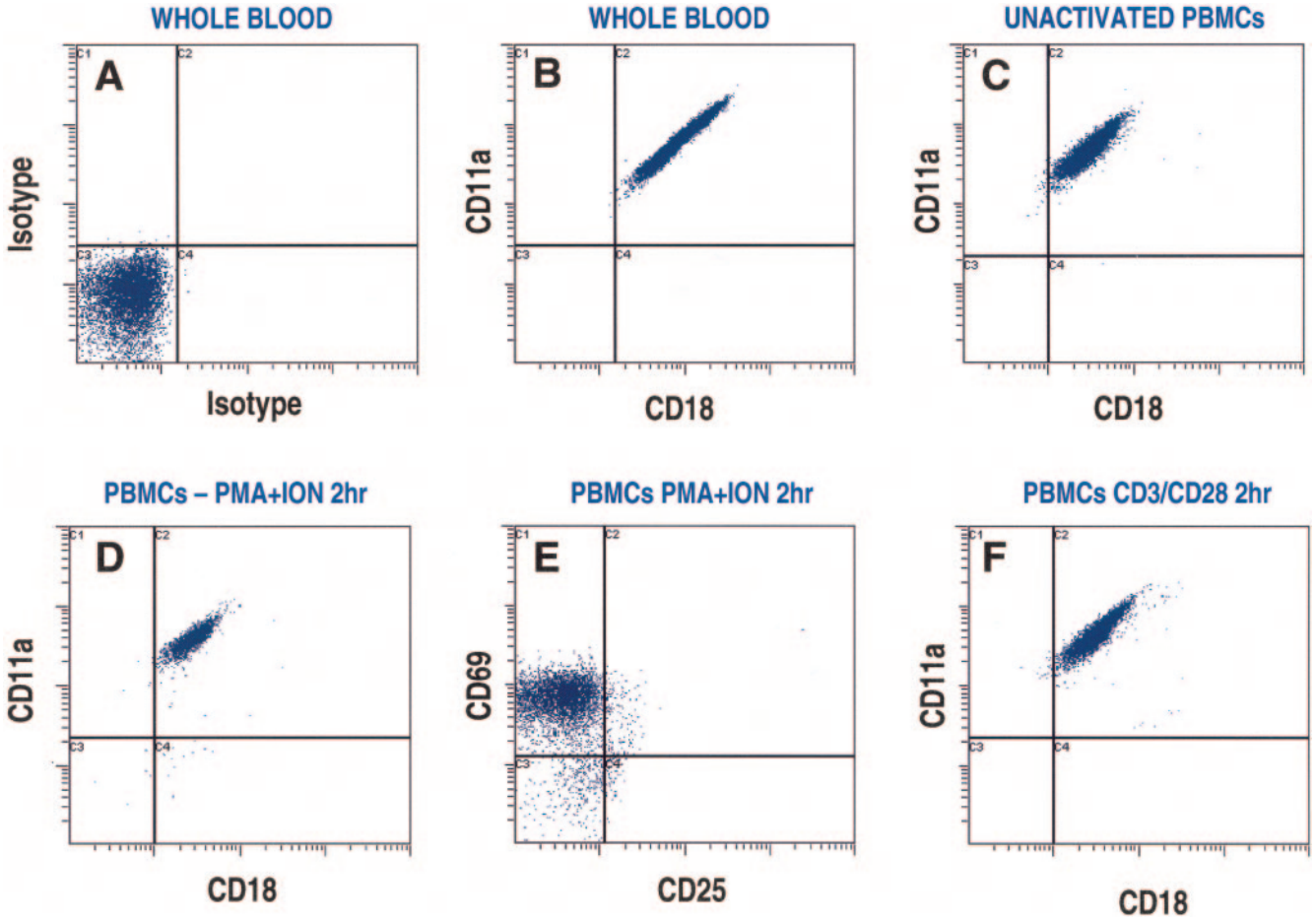

FIG. 2. CD11a and CD18 flow histograms. T-cell stimulation does not alter LFA-1 expression. Flow cytometry scatter plots representing the constitutive and culture-activated expression of LFA-1 on peripheral blood T cells are presented. Separate fluorescent antibodies for both the alpha chain (CD11a) and the beta chain (CD18) were stained with FITC and PE, respectively. All plots represent T-cell analysis following staining and gating of the T cells with CD3-PC5. (A) T-cell isotype control; (B) T-cell constitutive expression of LFA-1a and LFA-1b; (C) T-cell expression of LFA-1a and LFA-1b is unaltered following PBMC preparation and 2 h of culture; (D) T-cell expression of LFA-1a and LFA-1b is unaltered following PBMC stimulation with PMA-ionomycin for 2 h; (E) T-cell expression of CD25 and CD69 confirms T-cell activation (via CD69 expression) following PMA-ionomycin stimulation for 2 h; (F) T-cell expression of LFA-1a and LFA-1b is also unaltered following stimulation with anti-CD3 and anti-CD28 for 2 h.

Representative scatter plots are presented in Fig. 2. It should be noted that the simultaneous assessment of cell surface CD18 and CD11a (both chains of the LFA-1 molecule) presents an unusual histogram, similar to what would be expected from cytometry data with a spectral overlap-compensation problem (a diagonal fluorescence pattern). In fact, the histogram is accurate, with the unusual appearance the result of simultaneous assessment of the different chains of the same molecule by use of separate fluorochromes. Single-color and parameter control assessments were performed and verified that there was no issue with color overlap (data not shown). Simultaneous expression of CD69 and CD25 was assessed to verify T-cell activation. This finding is consistent with reports that LFA-1 undergoes a conformational change from a nonadhesive to an adhesive state upon T-cell activation, with no immediate change in the level of protein (5, 9). Culture durations were 2 h, which spanned the time during which the LFA-1 adhesive T-cell subset becomes functionally active.

Flow cytometry assessment of LFA-1-adhesive T cells. Creation of ICAM-1 beads yielded a substrate by which LFA-1 binding of adhesive T cells may be detected (by the resulting T cell-bead complexes) (Fig. 3). By using multicolor staining and a unique gating strategy, it was possible to resolve adhesive T cell-bead complexes from nonadhesive (free) T cells (Fig. 1). Bead binding to T cells resulted in a detectable increase in the side-scatter parameter for the adhesive T-cell subset. Since adhesion was detected by the scatter properties, all three of the fluorescent channels of the cytometer remained available for simultaneous analysis of surface markers. For routine analysis, an antibody combination that would allow resolution of T cells by immunoscatter (CD3 expression), tracking of monocytes (via CD14 expression, to verify noninterference), and CD69 expression (an extremely early marker of T-cell activation) was designed. The combinations were as follows: CD3-FITC, CD69-PE, and CD14-PC5. Figure 1 shows representative scatter plots that demonstrate T-cell gating, scatter property changes,

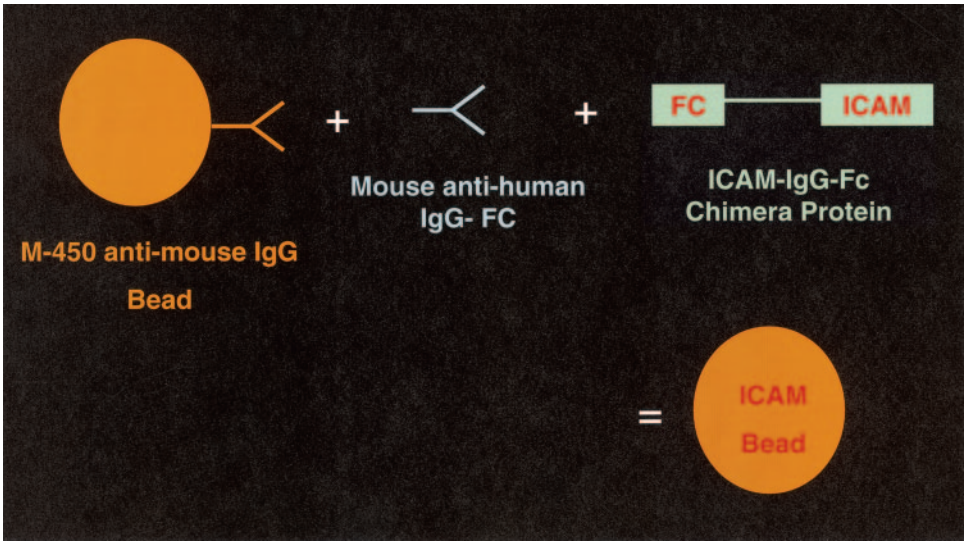

FIG. 3. Method of preparation of the ICAM-bead complexes. M-450 anti-mouse IgG beads (Dynal) were coated with mouse anti-human IgG Fc. An ICAM-1–human Fc chimera protein was then bound to the beads via the anti-human IgG. The result was ICAM-1-coated beads. The bead type was selected due to the availability of the anti-mouse IgG form and the ability to detect bead-T cell binding via side scatter alteration during flow cytometry analysis.

monocyte gating and tracking (red), quantitation of adhesive T cells, and CD69 expression in activated or unactivated PBMC cultures. Essentially, two gates were derived, one on the entire T-cell population via CD3 expression (Fig. 1C and H) and one on the monocyte population via CD14 expression (Fig. 1B and G). The monocytes were then colored for verification of noninterference (red in Fig. 1A and F), whereas the T cells were plotted alone versus the side scatter to quantify adhesion (Fig. 1D and I) or versus CD69 activation (Fig. 1E and J). The data show that the assay was specific, in that there was little binding between unactivated T cells and ICAM-1 beads (Fig. 1D), yet from activated T cells a distinct population of adhesive T cells clearly bound to the ICAM-1 beads (Fig. 1I). There was little binding between activated T cells and plain control beads (data not shown). Tracking of monocytes verified that no monocyte-related nonspecific binding interfered with the assay (Fig. 1A and C and Fig. 1F and H). It should be noted that the levels of activated T cells seen in the unactivated cell culture (Fig. 1E) represent constitutive levels, which may range from 2 to 10% in healthy individuals).

Kinetics of induction for LFA-1 adhesiveness. A kinetics analysis was performed by assessing T-cell adhesion to LFA-1 from 1 min to 3 h following PMA-ionomycin activation. The kinetics for the induction of LFA-1 adhesiveness (by detection of the levels of adhesive T cells) were extremely rapid, reaching levels near the plateau within 10 min. Figure 4 shows the kinetics plots for a single subject. The plots are representative of those for the assessments of three separate test subjects. The levels of adhesive T cells then tended to slowly decline over the subsequent hours. Simultaneous assessment of CD69 indicated that LFA-1 adhesiveness occurred much more rapidly than CD69 expression, which reached maximal levels following about 1 h of activation. The plotting of T cell-bead binding on naked (ICAM-1-negative) beads allowed an assessment of the experimental background and was found not to rise above minimal baseline levels throughout the time course (labeled "control beads" in Fig. 4).

Microscopy assessment of T cell-bead complexes. Electron microscopy and fluorescent microscopy were performed to verify the integrity of the activated T cell-bead complexes. Electron microscopy images demonstrated that there was indeed legitimate binding between the T cells and the ICAM-1 beads (Fig. 5A). For fluorescent microscopy images, both chains of the LFA-1 molecule (CD18 and CD11a) were labeled with

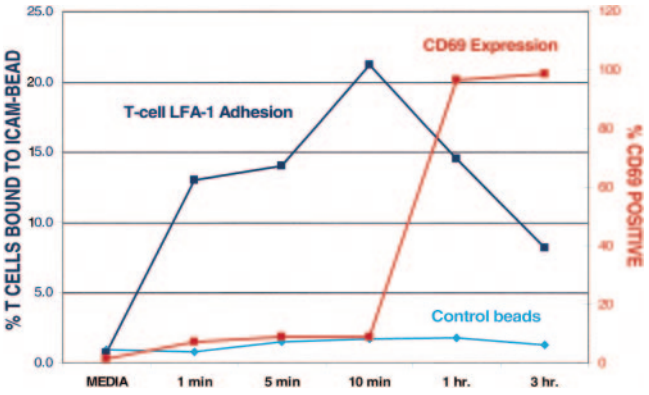

FIG. 4. LFA-1 and PMA-ion kinetics. Representative data from a single test subject demonstrating the kinetics of induction of LFA-1 adhesion versus the induction of CD69 expression on activated T cells. T cell–LFA-1 adhesion (dark blue) and CD69 expression (red) were detected by flow cytometry, as described in the text. The levels of T-cell binding to uncoated beads served as the control for nonspecific T-cell binding (light blue). The data are consistent with those observed following testing of blood from three healthy test subjects. Note that the induction of LFA-1 adhesiveness is detectable in as little as 1 to 5 min, well in advance of the induction of CD69 (considered an early marker for T-cell activation). The minimal level of binding to the control beads confirms that the T cell-bead binding is ICAM-1–LFA-1 specific.

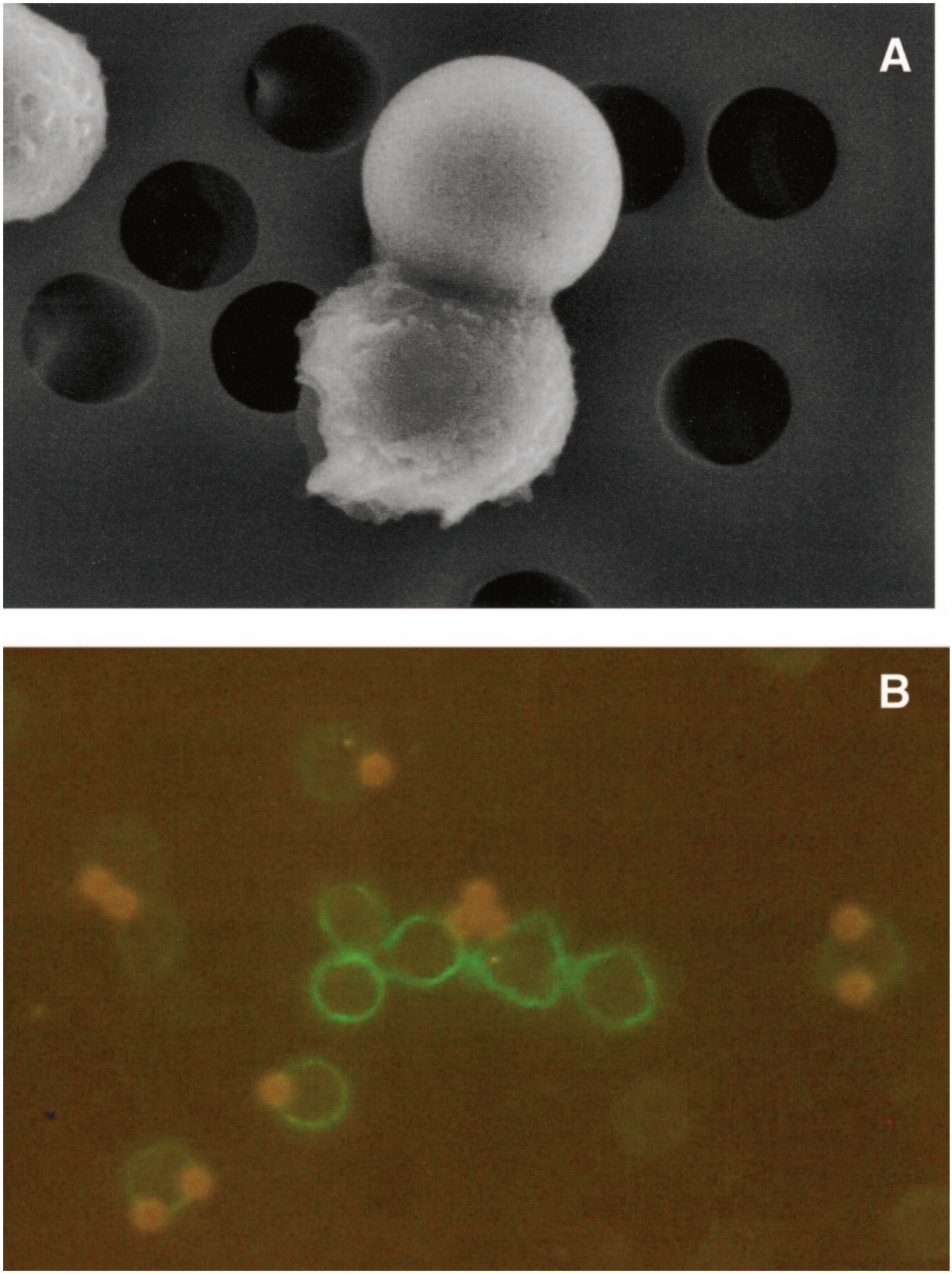

FIG. 5. Representative electron microscopy (A) and fluorescent microscopy (B) images depecting activated T cell–ICAM-1 bead binding. These images show that legitimate bead-cell binding occurs and confirm the cytometry data. The approximate sizes of the beads and T cells are 4.5 μm and 10 μm, respectively, on the fluorescent image (B). The T-cell size loss evident in the electron microscopy image (A) is a result of the sample preparation procedure, which includes dehydration. This process causes the cells to shrink, making their size appear closer to that of the M-450 beads.

primary antibodies. An Alexa Fluor 488 secondary antibody was used for fluorescence detection of LFA-1. Both free T cells and T cell-bead complexes, including cells coupled to both single and double beads, were readily visible by fluorescent microscopy (Fig. 5B). There was no obvious reorientation of LFA-1 toward the bead binding sites.

Phenotype of LFA-1 adhesive T cells. Since the detection of adhesive T cells occurs by using the scatter channels of the flow cytometer, complete phenotyping of the adhesive populations may be performed by use of the fluorescence channels. A phenotype analysis of the responding T-cell population was performed, and they were found to be a distinct and homogeneous population with respect to most of the surface markers assessed. The phenotype of the adhesive responding T-cell population was as follows: $CD3^+$ $CD4^+$ or $CD8^+$ $CD45RO^+$ $CD62L^+$ $CD27^+$ $CD57^-$ (data not shown). This phenotype is consistent with that expected from a previously activated memory T-cell subset.

DISCUSSION

There have been many studies of the biological function and immunological properties of leukocyte adhesion molecules. Problematic to the development of routine human assessments of LFA-1 activity is the fact that the molecule is constitutively expressed on most all peripheral T-cell subsets and that the levels of the LFA-1 protein do not change following T-cell activation. Assays for function (i.e., adhesion) instead of assays for the simple presence or absence of protein on the cell surface would have significantly more utility for research studies of adhesion molecules and immune function. In addition, simple assays that may be performed rapidly and that would yield reliable information might be applicable for clinical studies.

This study developed a simple and rapid flow cytometry-based assay for the detection of LFA-1 adhesion on peripheral blood T cells. This assay uses ICAM-1 beads as a substrate for the detection of adhesion, which is thus reliably detected by altered scatter properties in a positively identified cell population. The use of integrin substrate beads as a platform for the detection of cellular adhesion was first described in purified neutrophil cultures by Seo et al. (4). In that study, the authors used fluorescent beads to detect binding and a nucleic acid dye to identify neutrophils. In the method described here, the use of the magnetic beads and scatter properties to detect T-cell binding and adhesion leaves fluorescent channels free for simultaneous immunophenotype studies. Also, the simultaneous assessment of surface markers (including T-cell gating) as described here allows the use of this assay for heterogeneous mixed-cell cultures, and the assay allows various cell-cell interactions.

The authors suggest that for routine assessments, CD14 and CD69 be simultaneously assessed to track monocyte interference and T-cell activation, respectively, although neither assessment is absolutely required. The data revealed that following T-cell activation in culture, a distinct subset of T cells became adhesive to integrin binding, whereas other T cells clearly did not. Multicolor analysis revealed that the responding adhesive population was both homogeneous and distinct, with the phenotype corresponding to previously activated memory T cells. It should be noted that although LFA-1 is thought to be the most likely binding receptor on T cells, it cannot be excluded that other cell surface integrin molecules may also contribute to the T-cell binding of the ICAM-1 beads. This would not, however, alter the utility of the assay as a functional assessment of rapid integrin adhesion following T-cell activation. We suggest that cytometry-based assessments of integrin adhesion may be considered a marker for T-cell activation (for the responding T-cell subset) that occurs much faster than the movement of CD69 to the cell surface (Fig. 1). CD69 is currently considered a "rapid" indicator of T-cell activation, being transplaced to the cell surface within hours following activation.

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